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We are developing methods to derive gene transfer vectors capable of accomplishing targeted gene delivery to metastatic breast cancer cells. In this regard, strategies have been explored to modify adenoviral vectors by altering their binding tropism. Genetic methods employed have allowed for the modification of the native adenoviral binding protein (fiber) to incorporate cancer-relevant cell-binding ligands. Immunologic methods have yielded an antifiber antibody which specifically ablates native adenoviral tropism and provides a site for the addition of breast cancer-relevant ligands. The results developed herein have allowed for the successful retargeting of the adenoviral vector via either the genetic or immunologic approach. In addition, targeted, tumor-specific gene delivery has been achieved *in vitro*. These methods will now allow the evaluation of these vector systems in *in vivo* models of human breast cancer. The utility of the vectors in this context will allow the development of gene therapy strategies for disseminated breast cancer.

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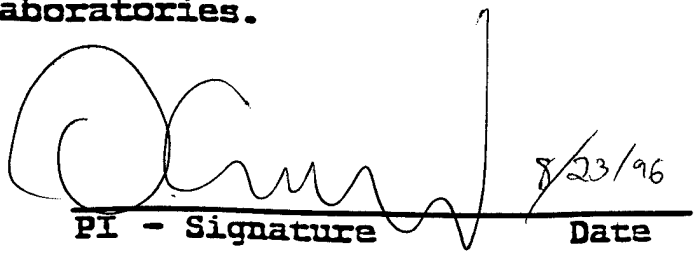

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David T. Curiel, M.D.

INTRODUCTION

It is the purpose of the present proposal to derive a gene transfer vector capable of specific and selective transduction of disseminated neoplastic breast cancer cells as a first step towards the development of gene therapy strategies for carcinoma of the breast. The development of a vector with this capacity would allow the targeting of malignant cells *in situ* after systemic delivery. Capitalizing on the unique capacity of recombinant adenoviral vectors to accomplish high efficiency *in vivo* gene delivery, it is our hypothesis that further modifications of this vector may be achieved to alter parent virus tropism such that selective transduction of malignant cells may be accomplished. These modifications would allow entry of the vector into receptor-mediated endocytosis pathways characteristic of the target breast cancer cells. To investigate mechanisms by which adenoviral tropism could be modified, several molecular strategies were employed. In this regard, the initial strategy to derive a tropism-modified recombinant adenovirus was directed towards genetic modification of the fiber protein to accomplish incorporation of heterologous cell-binding ligands, which could then mediate adenoviral entry via alternate receptor pathways. This approach capitalized on the knowledge that the endogenous cell-binding ligand of adenovirus was localized within the knob portion of the fiber protein. The aim was thus to localize the novel cell-binding ligand in the analogous position. This would accomplish two goals: 1) the novel cell-binding ligand would be localized in the region of the endogenous ligand, likely a propitious site as relates to interaction with the cognate cellular receptor; and 2) the novel cell binding ligand would be removed from other adenoviral capsid proteins, whose function might be important in distal, post-binding entry functions. The incorporation of heterologous peptides in the context of the fiber protein required consideration of the strict structural limitations of the fiber quaternary configuration. In this regard, the fiber protein is synthesized initially as a monomer (1). Upon localization to the nucleus, the molecular trimerizes by virtue of intramolecular, non-covalent interactions, initiated at the carboxy-terminus of the molecule. After trimerization, the amino terminus of the native fiber can then insert into the penton base (2). Thus, additions to the knob portion of the fiber, corresponding to the carboxy terminus of the molecule, could potentially impair trimer formation and thus prevent incorporation of chimeric fiber molecules into the mature adenoviral capsid. In addition to these considerations, it was important to achieve a final quaternary configuration whereby the incorporated ligand was localized on the exterior of the mature fiber trimer. Hence, it was not apparent a priori that added ligands would be localized outside the molecular structure of the knob and thus accessible to achieve target cell binding. With these considerations in mind, a strategy was undertaken to create fiber-ligand fusion proteins by genetically incorporating into the fiber gene heterologous sequences encoding peptides with physiologic ligand functions (Figure 1).

The initial analysis described in last year's progress report confirmed that: 1) the fiber fusion gene produced a chimeric fiber molecule capable of maturing into a normal trimeric quaternary configuration, and 2) the fiber fusion gene expresses a chimeric fiber whereby the heterologous ligand is localized on the exterior of the trimeric molecule. These studies thus demonstrated the feasibility of introducing heterologous peptide ligands into the cell-binding domain of the adenoviral fiber protein in a manner consistent with the ultimate derivation of a chimeric adenoviral particle.

BODY

A. <u>Construction of a fiber rescue system for generation of recombinant adenoviruses with modified fiber proteins</u>. After fiber binding, the tropism-modified virion must accomplish internalization, followed by gene transfer. These steps require the coordinated

functional actions of other adenoviral capsid proteins in mediating downstream entry events. Thus, it would be required at this juncture to incorporate the new fiber-ligand chimeras into viral particles for analysis of the gene transfer capacity of the composite particles. The most versatile method of construction of recombinant adenoviruses developed to date is recombination between a "rescue" plasmid containing an almost complete copy of the viral genome and a "shuttle" plasmid containing a foreign (or modified viral) gene flanked by surrounding regions of the Ad genome (3-5). Upon co-transfection and recombination *in vivo* between these two plasmids, a recombinant viral genome is generated. None of the shuttle and rescue plasmids constructed to date may be used for reconstruction of the fiber gene in the Ad5 genome. We thus undertook the development of an appropriate system for our purposes. The schema for this packaging system is described in Figure 2.

We sought to demonstrate that this packaging system could be employed to derive viral particles with variant fiber proteins allowing for alteration of cellular receptor tropism. In this regard, adenovirus serotypes 3 and 5 bind to target cells via distinct cellular receptors (6). We thus constructed a chimeric fiber gene whereby the tail and shaft domains were derived from the fiber of type 5 adenovirus and the knob domain derived from the fiber of type 3 adenovirus. This chimeric fiber gene was incorporated into the described fiber rescue system and employed to derive adenoviral vectors containing the variant fiber protein. Sequence analysis of the chimeric fiber gene predicted specific restriction endonuclease polymorphisms. Analysis of genomic DNA derived from the adenoviral vector containing the chimeric fiber protein, Ad5/3-Luc 3, confirmed the presence of these predicted polymorphisms. These findings confirmed that we had derived the adenoviral vector encoding the predicted chimeric fiber gene (data not shown).

The derived virus Ad5/3-Luc 3 thus contains a fiber gene encoding a chimeric fiber protein whereby the mature type 5 particle would be predicted to contain a fiber with a knob domain derived from adenovirus type 3. Such a virus should thus exhibit an altered receptor tropism such that target cell binding would occur via the type 3 adenovirus receptor rather than the type 5 receptor. To validate this concept, the recombinant adenovirus was delivered to HeLa cells, which contain receptors for both types 3 and 5 adenovirus. To show specificity of entry, competition experiments were performed employing recombinant type 3 and type 5 fiber knobs produced in *E. coli*. In this analysis, it could be seen that the type 5 parent virus, Ad5-Luc 3, achieved cellular entry via the type 5 receptor, as evidenced by specific blockade with the recombinant type 5 knob. In contrast, the adenoviral vector containing the chimeric fiber protein achieved cellular entry exclusively via the type 3 receptor (Figure 3). **These findings thus establish several key concepts with respect to our strategy. First, we have shown it is possible to incorporate modified fiber proteins into recombinant adenoviral vectors employing our two plasmid rescue system. In addition, these findings demonstrate that it is feasible to alter viral receptor tropism based upon genetic modifications of the fiber protein to alter its cellular recognition domains.**

B. <u>Definition of native adenoviral binding domain of fiber knob</u>. The second goal related to the achievement of tropism-modification is ablation of the ability of the fiber to recognize its endogenous native receptor. This step would eliminate non-tumor transduction as target cell entry would occur exclusively via the ligand introduced into the fiber. In this regard, a reasonable method to achieve this goal would be via mutagenesis to ablate domains of the fiber knob involved in native receptor recognition. Whereas native fiber knob has been crystallized and its structure partially characterized, the specific regions of the fiber knob involved recognition have not been defined (7). We thus sought to further localize the portion of the knob domain involved in receptor recognition as a first step to achieving functional ablation of these domains. As a method towards achieving this characterization,

we employed an immunologic strategy of domain mapping. To this end, we prepared monoclonal antibodies directed against the recombinant Ad5 fiber knob. This was accomplished by immunizing mice with intact adenovirus, followed by two rounds of immunization with purified recombinant fiber knob. Monoclonal anti-knob hybridomas were then generated by standard techniques in the UAB Hybridoma Core Facility. Supernatants from these hybridomas were then assayed for the following phenotypic characteristics: isotype, reactivity with recombinant fiber knob, and neutralization of adenoviral-mediated cytopathic effect (CPE). Isotype was determined by the commercially available IsoStrip assay (Boehringer Mannheim). Reactivity with recombinant knob was assayed by ELISA using plates coated with recombinant adenovirus type 5 knob. Extent of antibody binding was determined photometrically with an anti-IgG-alkaline phosphatase conjugated antibody. Neutralization of adenovirus type 5 cytopathic effect was determined by inhibition of adenovirus infection of HeLa cells by Ad5-Luc 3 (data not shown). From the ELISA data, it has evident that these antibodies bind knob protein with high affinity. In addition, as determined by the inhibition of infectivity of adenovirus, it was also evident that these antibodies are localized within a region of the knob domain essential for adenovirus binding. We next sought to utilize this data towards defining the native receptor recognition domain localized within the fiber knob. The specific epitopes of the anti-knob antibodies were identified by biopanning with a filamentous phage-displayed random hexapeptide library. This analysis was carried out in conjunction with our collaborator P. Boulanger (see letter of collaboration). This analysis defined three discrete neutralization domains: (i) near the shaft-knob junction (residues 388-420); (ii) within residues 440-510; and (iii) near the C-terminus (residues 541-570) (Figure 4).

We have thus demonstrated in this progress report that it is rational to pursue our strategy of tropism modification of the adenovirus for cell-specific gene delivery. In this regard, we have shown that we can derive fiber-ligand chimeras with structural features compatible with their incorporation into mature viral particles. We have further shown that we can derive adenovirus particles containing variant fiber proteins. Most importantly, we have shown that the incorporation of these variant fibers can alter viral tropism. Finally, we have defined the potential sites of the native receptor recognition domain within the fiber knob. This information will provide a rational foundation for steps directed at ablating native tropism employing mutagenesis techniques. This data thus establish the overall feasibility of our genetic strategy to alter the tropism of adenovirus for purposes of tumor cell-specific targeting with adenoviral vectors.

C. Immunological strategies to alter adenoviral tropism for cell-specific targeting. As an additional method to alter adenoviral tropism, we have pursued immunologic methods. This strategy has demonstrated feasibility in the context of retargeting of retroviral vectors (8). In addition, this method would offer advantages of both a theoretical and practical nature. From the theoretical standpoint, the immunologic method offers the potential for a single-step retargeting event whereby both native tropism would be ablated and novel tropism conferred by virtue of a single modification strategy. In this regard, anti-knob antibodies could serve to ablate native cell recognition domain as well as serving as the locus for addition of targeting moieties such as ligands or anti-receptor antibodies directing the virus to alternate entry pathways. **From the practical standpoint, anti-knob antibody-ligand conjugates could be rapidly derived with the potential to screen many candidate cellular entry pathways for utility in our breast cancer retargeting strategy**. We thus initially employed a chemical linkage strategy as proof of principle with respect to this approach. To this end, we hypothesized that it would be possible to ablate endogenous adenoviral tropism by employing a neutralizing anti-knob monoclonal antibody, and that novel tropism could be introduced by conjugating a cell-specific ligand to this mAb or an antibody fragment. To test this concept,

we chose to target the high affinity folate receptor (K_d 10^{-9} M), which is overexpressed on the surface of several malignant cell lines, including ovarian, lung and breast carcinomas and brain tumors (9-13). In addition, several authors have demonstrated that folate conjugates can be employed to deliver macromolecules, including DNA and protein, specifically to folate receptor-bearing cells (14-21). This concept is illustrated schematically in Figure 5.

D. Generation and analysis of Fab fragment of neutralizing anti-knob antibody. The neutralizing mAb, designated 1D6.14, was chosen for further study on grounds of its high affinity for knob and strong neutralizing of Ad infection. We rationalized that for our purposes of developing a targeted adenoviral vector by immunological methods, it would be preferable to employ the Fab fragment of the anti-knob antibody (1D6.14), rather than the intact immunoglobulin. In this manner, we sought to prevent the two antigen-binding arms of the parent antibody crosslinking different viruses to form large complexes which might prove refractory to cellular uptake. Therefore intact 1D6.14 was digested with papain and the Fab fragments were collected in fractions as the flow-through of a protein A agarose affinity chromatography column to which the Fc fragments and undigested IgG bound. Protein-containing fractions were identified by determination of the absorbance at 280 nm and then subjected to SDS-PAGE. This analysis revealed that the Fab fragments were of the correct molecular weight and were electrophorectically pure (data not shown). Fractions containing the Fab fragment were pooled and the protein concentration was determined. The Fab fragment of anti-knob mAb 1D6.14 was then analyzed to verify that it retained the ability of the parent molecule to neutralize adenovirus infection. The recombinant serotype 5 adenoviral vector AdCMVLuc, which carries the firefly luciferase reporter gene, was premixed with varying dilutions of either the intact parent immunoglobulin or the Fab fragment prior to infection of HeLa cell monolayers. Twenty-four hours post-infection, the expressed luciferase activity was determined: this value represents an indirect measurement of the number of infecting viruses. Preliminary studies showed that both the parent antibody and the Fab fragment were capable of neutralizing adenovirus infection in a dose-dependent manner, whereas an irrelevant control antibody failed to block infection (data not shown).

E. Construction and characterization of Fab-folate conjugate. Having confirmed that the Fab fragment of anti-knob mAb 1D6.14 could block Ad infection, we sought to introduce the second requirement for a targeted adenoviral vector: the ability to recognize specific receptors expressed on the surface of the target cells. For this purpose we chose to construct a conjugate of the vitamin folate and the Fab fragment of the neutralizing anti-knob mAb, with the aim of targeting adenoviruses to the high-affinity folate receptor which is overexpressed on a number of malignant cell lines. Previous studies have demonstrated that folate can be conjugated via its γ-carboxylate group to a variety of macromolecules, including antibodies, without losing affinity for its cellular receptor (14-21). Since folate and folate-macromolecule conjugates are internalized by the folate receptor by a mechanism termed potocytosis (22) which involves nonclathrin-coated caveolae with a diameter of 60 nm, an adenovirus (diameter 65-80 nm, excluding the fibers) would be too large to enter this pathway. However, we hypothesized that after binding specifically to the cell surface folate receptors, the adenoviral vector would still be able to accomplish internalization by its native endocytotic pathway (23-26) mediated by the interaction of the penton base with secondary host cell receptors, α v integrins (27,28). The Fab fragment of the neutralizing anti-knob antibody, 1D6.14, was covalently coupled with folate. The resulting conjugate, hereafter referred to as the Fab-folate conjugate, was characterized both structurally and functionally. The conjugation of folate to the antibody fragment was verified by SDS-PAGE under denaturing conditions followed by immunoblot analysis employing an anti-folate mAb. An alkaline phosphatase-conjugated secondary antibody specific for the Fc region of mouse IgG was used to prevent cross-reaction with the Fab fragment of 1D6.14. It could be shown that

the antifolate antibody reacted specifically with the Fab-folate conjugate, while failing to recognize the unconjugated Fab fragment, thus confirming the success of the conjugation (data not shown).

The ability of the Fab-folate conjugate to recognize the folate receptor was evaluated in a competition binding assay using ^{3}H-labeled folate and KB cells, a folate receptor-positive human nasopharyngeal carcinoma cell line, KB (29). This demonstrated that binding of the labeled folate to KB cells was inhibited by the Fab-folate conjugate and by a conjugate of folate with the intact 1D6.14 antibody, but not by the antibody alone (data not shown). Thus the conjugation of folate to the Fab fragment of the neutralizing antibody had not destroyed the ability to bind the folate receptor, a finding which is in accord with previous reports in the literature. We then determined whether the conjugation of folate to the neutralizing anti-knob Fab fragment had affected its ability to block adenovirus infection. AdCMVLuc was premixed with various concentrations of the Fab-folate conjugate prior to infection of HeLa cell monolayers. Expression of luciferase activity was determined 24 hours post-infection (data not shown). The Fab-folate conjugate was confirmed as being capable of neutralizing adenoviral infection in a dose-dependent manner.

F. Targeting of AdCMVLuc to folate receptor. Having verified the reagents, we then proceeded to test our hypothesis that the Fab-folate conjugate would be capable of modifying the tropism of an adenoviral vector to permit the specific targeting of the folate receptor. The adenoviral vector AdCMVLuc was premixed with the optimal neutralizing concentrations of the unconjugated Fab fragment or the Fab-folate conjugate prior to infection of KB cell monolayers maintained in folate-free medium. The level of luciferase activity was determined 24 hours post-infection. As shown in Figure 6, the unconjugated Fab fragment blocked infection of KB cells by AdCMVLuc by preventing the knob domain of the virus fiber from binding to its cellular receptor. However, a high level of luciferase activity was restored when AdCMVLuc was premixed with the Fab-folate conjugate, indicating that the re-targeted virus was capable of efficient infection. When a competition experiment was performed in which the target cells were preincubated in folate-containing medium and the infection carried out in the presence of excess free folate, the Fab-folate conjugate failed to mediate infection of KB cells by AdCMVLuc-the free folate saturated the target receptor, preventing the binding of the viral complex. This demonstrates that the Fab-folate conjugate was capable of redirecting adenoviral infection of target cells specifically via the folate receptor.

Thus, we have demonstrated the feasibility of employing an immunologic approach to re-target adenoviral vectors. This methodology will allow rapid and facile definition of cellular ligands compatible with our adenoviral retargeting strategies.

CONCLUSIONS

Two distinct strategies to alter adenoviral tropism for tumor cell-specific gene delivery have been developed. The feasibility of genetically modifying the adenoviral fiber protein has been established and methods to derive genetic fiber ligand chimeras developed. In addition, the basis for immunologic retargeting of adenoviral vectors has been demonstrated. Employing this method, targeted-specific gene delivery to tumor cells has been achieved. This significant progress will shortly allow analysis of targeted gene delivery to breast cancer cells in the context of murine models of disseminated carcinoma of the breast.

FIGURES AND FIGURE LEGENDS

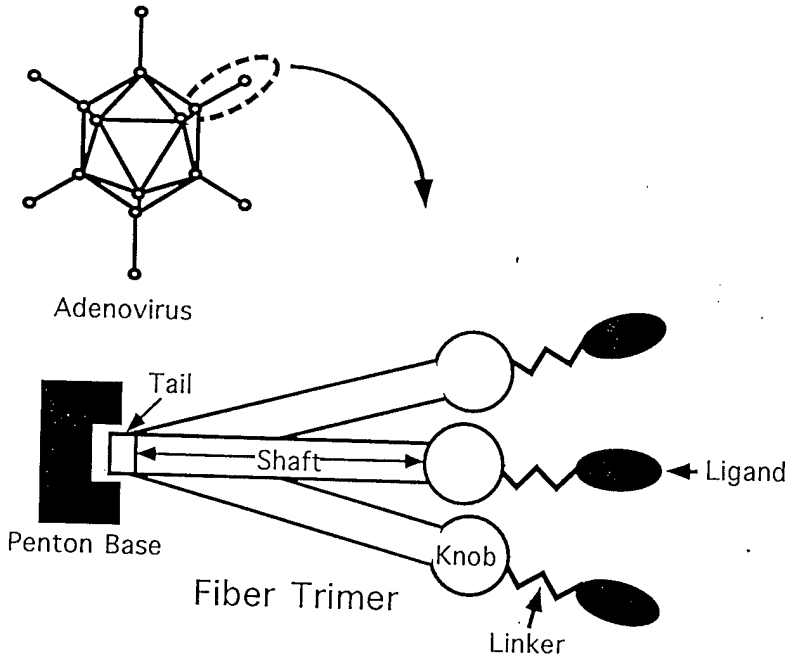

Figure 1. Strategy to incorporate heterologous peptide ligand at the knob domain of adenoviral fiber. The strategy presented herein involves the genetic modification of the fiber gene to generate a fiber chimera consisting of mature trimeric fiber, peptide linker, and an added physiologic ligand at the knob domain. This ligand is designed to target the adenovirus into heterologous cellular entry pathways.

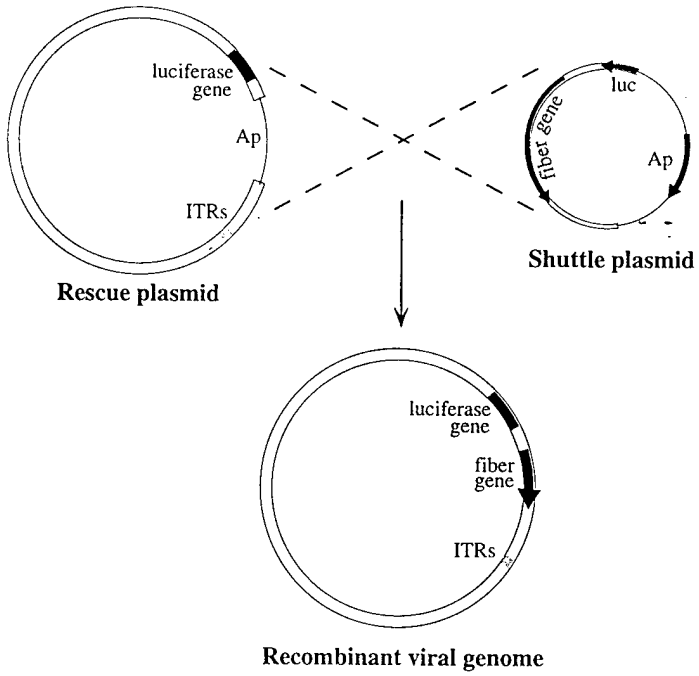

Figure 2. Schema for generation of adenoviral vector containing fiber variants employing *in vivo* homologous recombination. Recombination between the fiber rescue plasmid, pVK5, and the shuttle plasmid, would be predicted to yield the depicted recombinant adenoviral genome. This recombinant genome would contain the fiber variant gene originating from the shuttle plasmid.

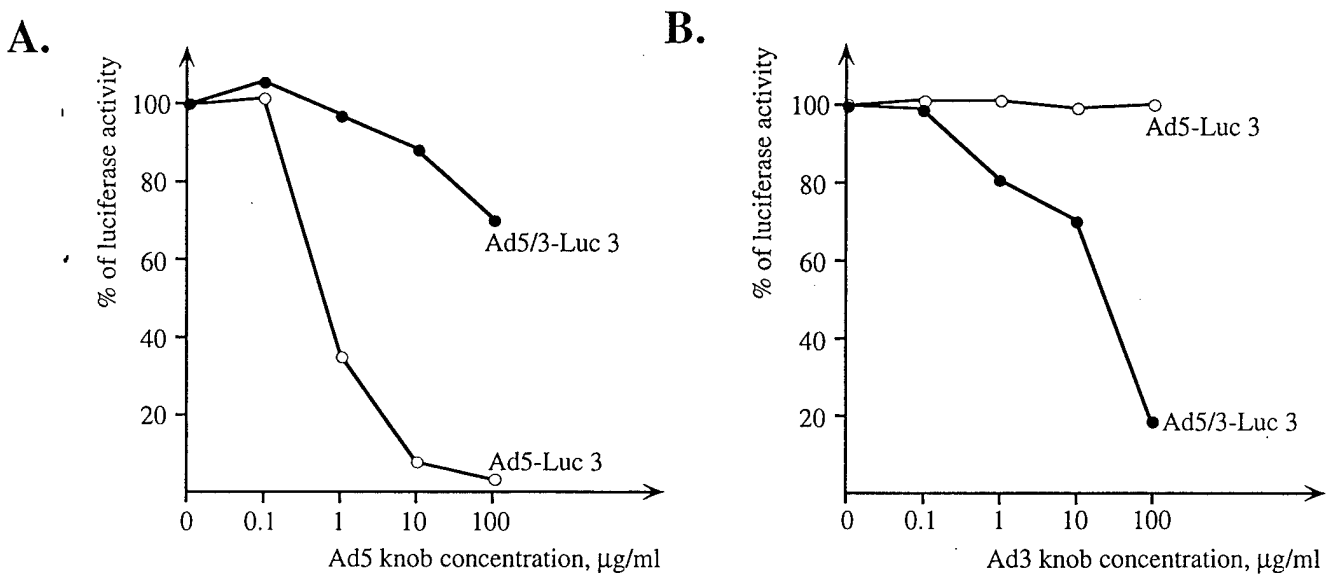

Figure 3. Gene transfer mediated by the adenoviral vectors Ad5-Luc 3 and Ad5/3-Luc 3. **A.** Infection of HeLa cells via the adenovirus type 5 receptor employing the adenovirus type 5 derivative Ad5-Luc 3. Competition with recombinant type 5 fiber knob is demonstrated. **B.** Infection of HeLa cells via the adenovirus type 3 receptor employing the chimeric adenovirus Ad5/3-Luc 3. This adenoviral vector is of type 5 derivation but contains a chimeric fiber protein incorporating a fiber knob domain derived from adenovirus type 3. Competition with recombinant type 3 fiber knob and type 5 fiber knob is demonstrated.

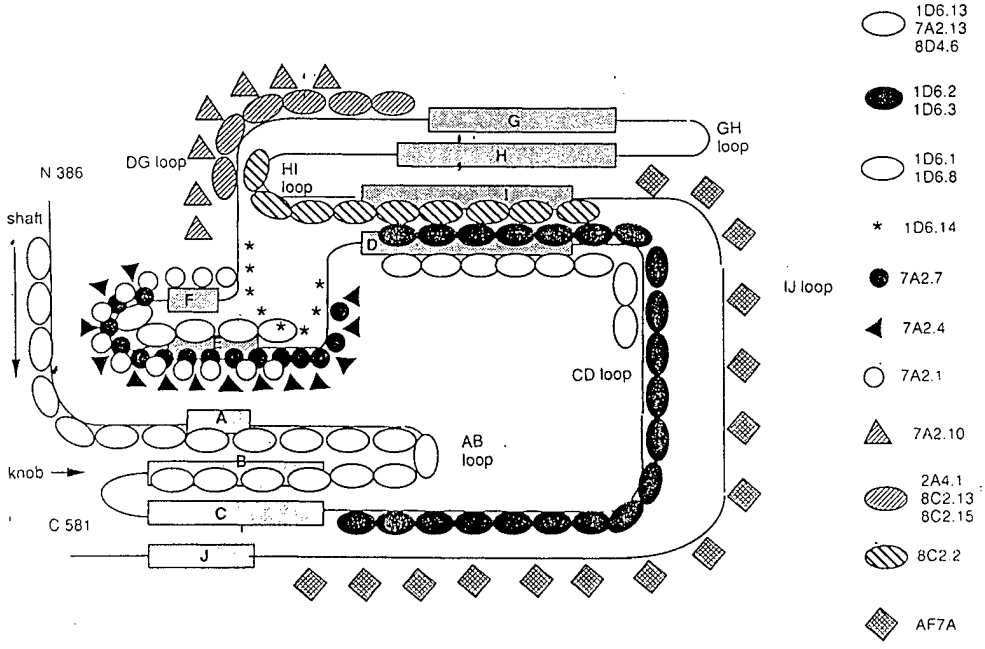

Figure 4. Anti-knob antibody recognition sites within predicted fiber knob structure.

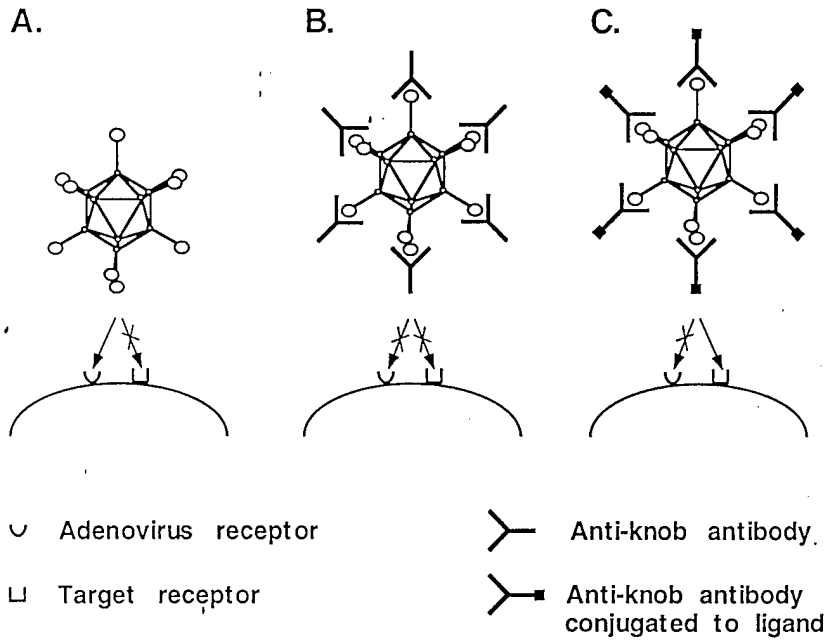

Figure 5: Strategy for immunological retargeting of adenoviral vector. **A.** Adenoviral attachment to cells is accomplished by the high affinity binding of the knob domain of the fiber to an as yet unidentified membrane surface receptor. **B.** When complexed with a neutralizing antibody directed against the knob domain, the adenovirus is unable to bind to its cellular receptor. **C.** Conjugation of a cell-specific ligand to the neutralizing antibody is hypothesized to permit binding to a novel target receptor on the cell surface.

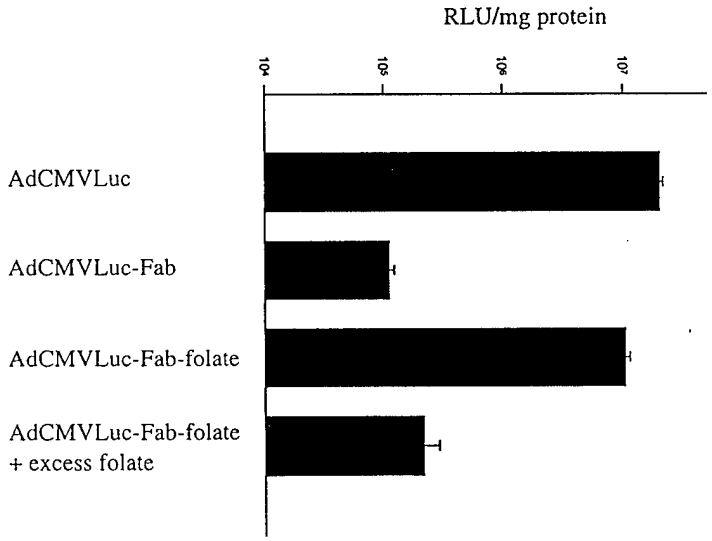

Figure 6: Redirection of adenoviral infection mediated by the conjugate of folate with the Fab fragment of neutralizing mAb 1D6.14. An optimal dose of Fab or Fab-folate was incubated with a replication-incompetent adenoviral vector encoding the firefly luciferase gene (AdCMVLuc) at room temperature in a total volume of 20 µl HBS. After 30 minutes, the complexes were diluted to 1 ml with folate-free RPMI 1640 + 2% FCS and added in triplicate to 6-well plates containing 80% confluent KB cells which had been washed with PBS. Prior to infection, the target cells had been passaged twice in folate-free RPMI. After incubation for 24 hours at 37°C, the cells were lysed and extracts assayed for luciferase activity as described.

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